Functional Domains of the Epithelial Sodium Channel

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The epithelial sodium channel (ENaC) plays an important role in ion transport of many organ systems. In kidney, colon, and sweat gland, ENaC-mediated transepithelial sodium transport helps to adjust sodium excretion in the urine, feces, and sweat to fulfill homeostatic requirements. In lung and airways, ENaC activity is important for alveolar liquid clearance and regulation of mucous fluidity. In the taste buds of the tongue, ENaC is likely involved in salt tasting, whereas ENaC expression in the eye and inner ear may help to control the ionic composition of the aqueous humor and the endolymph, respectively. These diverse functions of ENaC (1) require intricate molecular regulatory systems that precisely adapt ENaC function to constantly changing electrolyte transport requirements. The best studied regulatory system is the aldosterone-dependent regulation of ENaC in the kidney, which is of major importance for the maintenance of whole-body sodium homeostasis and for BP control (2). This review focuses exclusively on the role of ENaC in renal sodium handling. Distribution and aldosterone-dependent regulation of ENaC in the kidney *in vivo* is discussed briefly, and the functional domains of ENaC that are important for the regulation of the channel under physiologic and pathophysiologic conditions are highlighted.

ENaC and the Aldosterone-Sensitive Distal Nephron

ENaC is the major apical sodium entry pathway present in the aldosterone-sensitive distal nephron (ASDN). The ENaC-mediated sodium transport across the apical membrane of tubule cells is electrogenic, depolarizing this membrane and favoring K^+ secretion *via* apical K^+ channels such as ROMK. Moreover, sodium transport across ENaC may osmotically drive transepithelial water transport *via* vasopressin-dependent apical water channels (*i.e.*, aquaporin-2). As such ENaC in the ASDN plays a pivotal role in the final adjustment of renal sodium, potassium, and water excretion. The importance of ENaC for sodium, potassium, and fluid homeostasis is emphasized by the observation that ENaC gain-of-function mutations or loss-of-function mutations lead, respectively, to extracellular volume expansion (Liddle's syndrome or pseudohyperaldosteronism) or to renal salt-wasting syndromes (pseudohypoaldosteronism type 1) associated with alterations in potassium homeostasis (2,3).

Morphologic and functional studies on rodent and human kidneys (4–6) indicated that at least three successive tubule portions—the late portion of the distal convoluted tubule (DCT), the connecting tubule (CNT), and the collecting duct (CD)—contribute to the ASDN. Although these segments have distinct structural and functional features (6), they share in common the expression of ENaC, the mineralocorticoid receptor (MR), and the 11-β hydroxysteroid dehydrogenase type 2 proteins (5). The latter confers mineralocorticoid-selectivity to the MR by rapid metabolization of circulating glucocorticoids. It is uncertain whether the early DCT also belongs to the ASDN. The early DCT weakly expresses the MR but lacks detectable 11-β hydroxysteroid dehydrogenase type 2 (7). In the kidney, aldosterone increases the protein expression of the DCT-specific NaCl co-transporter that is sensitive to thiazides (NaCl co-transporter or thiazide-sensitive co-transporter) (8,9). Consistently, aldosterone has been shown to stimulate electroneutral Na^+ transport in the DCT (10). It needs to be determined whether these stimulatory effects occur along the entire DCT or only in the late DCT.

The subcellular localization of ENaC along the axis of the ASDN changes drastically with the elevation of plasma aldosterone levels in response to changes in the sodium diet (11,12). In rodents that are kept under a high dietary sodium intake with low plasma aldosterone levels, ENaC subunits are barely detectable at the luminal membrane and are found almost exclusively at intracellular sites, the identity of which remain to be identified. On a standard dietary sodium intake (European laboratory diet) with moderate plasma aldosterone levels, ENaC subunits are traceable at the luminal membrane of late DCT and early CNT. However, in segments farther downstream (late CNT and CD), particularly β- and γ-ENaC subunits remain almost exclusively localized at intracellular sites. Under a low dietary sodium intake with high plasma aldosterone

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levels, ENaC becomes detectable in the luminal membrane along the late DCT, CNT, and CD (11,12). Nevertheless, the axial gradient for apical ENaC still prevails, and the apical localization of ENaC subunits is more prominent in early ASDN than in late ASDN (Figure 1). This immunohistochemically traceable axial gradient of apical ENaC localization is corroborated by a recent series of elegant patch-clamp studies (13). ASDN segments that were isolated from animals that were on a standard US laboratory diet exhibited no amiloride-sensitive currents at the single channel level, whereas CNT and CD that were isolated from rats with elevated plasma aldosterone levels revealed significant single-channel ENaC currents. In general, amiloride-sensitive currents decreased in the following order: CNT > initial CD > CD (13). These findings on apical ENaC localization and activity are consistent with previous studies on microperfused rat tubules (14,15) and on isolated rabbit tubules (16) that established several times higher sodium transport rates in early ASDN (DCT and CNT) than in ASDN segments farther downstream (CD), and also with studies that showed a progressive decrease of the basolateral Na-K-ATPase activity along the ASDN (17). Taken together, the data clearly suggest that the aldosterone-dependent adaptation of renal sodium excretion to dietary sodium intake occurs predominately in the early ASDN, whereas the late ASDN gets recruited only under high plasma aldosterone levels. The importance of the early ASDN *versus* late ASDN for the maintenance of sodium balance was recently highlighted by the development of a mouse model with targeted inactivation of α-ENaC exclusively in the CD. These mice survive well and are able to maintain sodium and potassium balance, even when challenged by salt restriction or potassium loading (18).

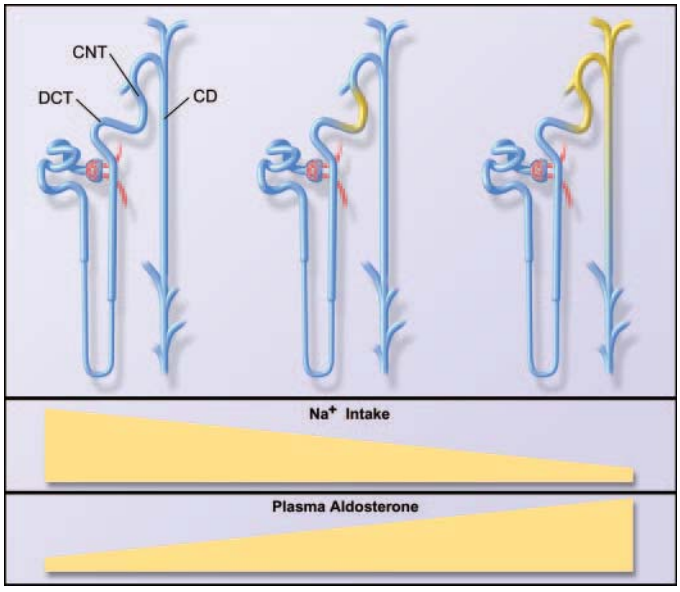

Figure 1. Apical localization of epithelial sodium channel (ENaC) along the aldosterone-sensitive distal nephron. On a high dietary sodium intake, ENaC subunits are almost absent from the cell surface and reside exclusively at intracellular sides in the aldosterone-sensitive distal nephron (ASDN) segments (the late distal convoluted tubule [DCT], the connecting tubule [CNT], and the collecting duct [CD]). Lowering dietary sodium intake progressively increases the cell surface abundance of ENaC starting in the early ASDN (*i.e.*, late DCT and early CNT) under moderate Na^+ intake and extending into the late ASDN under more severe Na^+ restriction. Shading of the tubules corresponds to the amount of apical ENaC and indicates the axial gradient of ENaC cell surface abundance along the ASDN. Illustration by Josh Gramling—Gramling Medical Illustration.

Functional Properties

Electrophysiologic investigations of ions transport first in the toad skin and in toad urinary bladder, then in isolated perfused distal nephrons have identified large amiloride-sensitive electrogenic Na^+ currents stimulated by aldosterone (19). The first recordings of amiloride-sensitive Na^+ currents at the single-channel level were obtained from principal cells of microdissected cortical CD and from cell lines and revealed the functional signature of the epithelial sodium channel (20,21). This channel was highly selective for Na^+ over K^+ ions (P_{Na}/P_K >100); the single-channel conductance was 4 to 5 pS with Na^+ as the charge carrier and 9 to 10 pS with Li^+ ions. From patch-clamp recordings, ENaC usually shows long open and closed times. The open probability (Po) of ENaC, however, is variable under similar physiologic conditions and range from ≤0.05 and >0.95; this variation in Po could reflect different gating modes of the channel (22). Switch between gating modes can be influenced by changes in the membrane voltage, although the response to voltage is relatively weak compared with the classical voltage-gated ion channels. Other variables have been proposed to affect ENaC gating and include intracellular pH and Ca^{2+} and hormones (23,24). High extracellular concentrations of Na^+ ions tend to inhibit ENaC activity, a phenomenon called self-inhibition (25). This fast inhibition of ENaC upon increasing extracellular Na^+ concentration is not related to changes in intracellular Na^+ concentration and is strongly dependent on the temperature.

From a pharmacologic point of view, ENaC is blocked by submicromolar concentrations of amiloride. Amiloride is a weak base, and the pH dependence of the block suggests that the ionized form of amiloride is efficient. Consistent with this notion, the amiloride block is voltage dependent, a characteristic that is commonly found for charged blockers binding in the channel pore within the transmembrane electric field (26). ENaC inhibition by amiloride is also dependent on the external concentration of Na^+ ions, and experiments in toad bladder are consistent with a competitive interaction between the permeating Na^+ ion and the blocker (27). These observations suggested overlapping binding sites for amiloride and Na^+ ions in the external pore vestibule of the channel.

The primary structure of ENaC was identified by expression cloning in *Xenopus* oocytes. ENaC is a heteromultimeric channel that is made of three homologous α, β, and γ subunits that share approximately 30% homology at the amino acid level (28,29). The expression of the $\alpha\beta\gamma$-ENaC subunits recapitulates the functional and the pharmacologic properties of the channel (29). Predictions from the primary structure are consistent with the presence of two transmembrane α helices. The large loop

that separates the two transmembrane domains is facing the extracellular side as shown by the glycosylation pattern of the protein, although hydrophobic segments within this putative extracellular loop might be embedded in the membrane (see Figure 2) (30). According to these predictions, the N- and C-termini are facing the cytosolic side of the membrane. Most of the reports on the subunit stoichiometry of members of the ENaC/degenerin channel family agree with a four- subunit architecture, and in the case of ENaC, the channel is likely made of 2α, 1β, and 1γ subunits (31–34). The issue of the subunit stoichiometry is not solved because ENaC channels with up to nine subunits have been proposed (35). From our knowledge of three-dimensional structure of different ion channels, such a nonameric structure, is unusual for highly selective ion channels. Such a high number of subunits forming the channel complex may simply reflect clustering of several ENaC channels at the cell surface.

Structure and Function Relationships

The Channel Pore

The recognition of amino acid residues on α-, β-, and γ-ENaC subunits that when mutated drastically change the channel affinity for the pore blocker amiloride (up to a 1000-fold increase in amiloride Ki) (36), identified a sequence (αS583, βG525, and γG537 in the rat ENaC sequences) that likely forms the outer vestibule of the channel where amiloride binds (see Figure 2). These amino acids are located at the extracellular start of the second transmembrane α helix; mutations within this region affect the amiloride-blocking kinetics, indicating that the mutated amino acids participate in the close interaction between amiloride and the channel pore, *i.e.*, are part of the amiloride binding site (37).

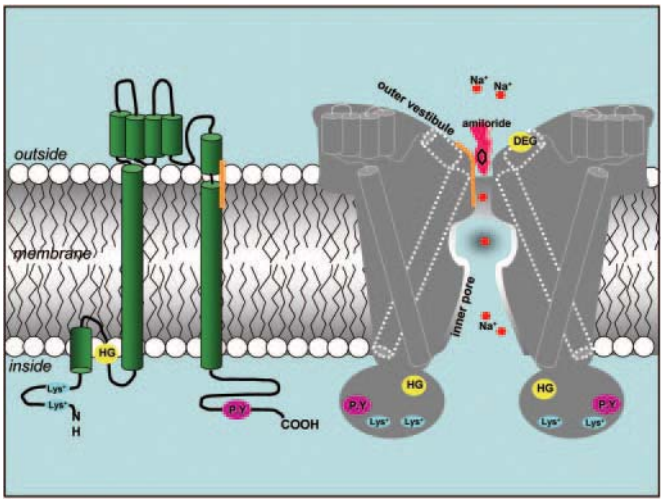

Figure 2. Schematic representation of the ENaC channel in the membrane. (Left) Secondary structure predictions and membrane topology of one ENaC subunit. Each subunit is made up of two transmembrane segments, TM1 and TM2. Cylinders represent putative α helices. P, extracellular pore helix; HG, conserved His and Gly residues among all of the members of the ENaC/degenerins family members; P.Y, PY motif common to the $\alpha\beta\gamma$-ENac subunits; DEG, degenerin site in the P-helix. (Right) ENaC channel model. The external pore vestibule is made up of the P-α helix, the linker between P and TM2 helix that composes the amiloride binding site. The first residues of TM2 participate in the ion selectivity filter that presents the narrowest part of the ion conduction pathway, where the permeant ion is in its dehydrated form. Structures that line the internal water-filled pore remain to be identified. Illustration by Josh Gramling—Gramling Medical Illustration.

It is likely that other regions in the ENaC subunit sequence also influence channel affinity for amiloride, but these regions remain to be identified. For instance, mutations in the WYRFHY sequence lower the affinity for amiloride of a 21-pS channel made of α-ENaC subunits exclusively (38,39). There is no evidence that these mutations affect the block by amiloride of the native 5-pS channel made of $\alpha\beta\gamma$ subunits and thus participate in the amiloride binding site in the native channel.

According to an early model of the ENaC pore based on the interaction between pore blockers, large blocking cations and the permeating Na^+ and Li^+ ions, it was proposed that the external vestibule of ENaC resembles a funnel-like structure that narrows from the amiloride binding site down to the selectivity filter, allowing only small permeant ions to pass through the channel pore (40). Consistent with this model, mutations of conserved Gly and Ser residues downstream of the amiloride binding site in the rat α-ENaC sequence (GSS sequence) and located within the first five amino acid residues of the second transmembrane α helix change the permeability properties of the channel (41–43). First, these mutations allow larger cations to pass through the channel but also reduce the unitary conductance of ENaC, *i.e.*, the flux of Na^+ or Li^+ ions through the pore. These experiments indicate that these mutations disrupt the channel structure at a site where the permeating ions are in close contact with the channel pore, likely the selectivity filter.

Thus, the stretch of seven amino acid residues at the external start of the transmembrane α helix that compose the amiloride binding site and the selectivity filter line the external entry of the channel pore. The structures that line the internal pore and its opening into the cytosol remain to be identified.

Channel Gates

Secondary structure predictions reveal a short external α helix (pore helix) upstream of the second transmembrane segment (Figure 2). Within this external vestibule helix, a mutation of a conserved Ala residue in degenerin channels causes degeneration of touch receptor cells in *Caenorhabditis elegans* with morphologic features that are consistent with an abnormal cation leak into the cell (44). At the corresponding position in α-ENaC, introduction of a cysteine for a conserved Ser (αrENaC S576) and its covalent modification with sulfhydryl reagent induces changes in the ENaC gating with extremely long channel opening dwell times ($Po > 0.9$) (45,46). It is interesting that the Cys at this position 576 is accessible by the sulfhydryl reagent only when the channel is open. These observations are consistent with the presence of an extracellular gate controlling the channel openings. The putative external vestibule helix may function as an external gate and the sulf-

hydryl reagent as a "foot in the door" that locks the channel in the open conformation.

Evidence for an intracellular gating domain first came from genetic analysis of ENaC loss-of-function mutations associated with pseudohypoaldosteronism type 1. It was found that in one family, the disease results from a mutation of a highly conserved His-Gly motif in the N-terminus of the β-ENaC subunit (47). Corresponding mutations in the α-ENaC subunit modifies channel gating by shortening the channel mean open time and therefore decreasing the Po (48). This His-Gly motif that is conserved among all of the members of the ENaC/degenerin channel family is located in an intracellular linker between a putative hydrophobic α helix and the first transmembrane segment. Furthermore, this region of the N-terminus in the $\alpha\beta\gamma$-ENaC subunits is rich in cysteine residues. These residues are responsible for the high ENaC sensitivity to inhibition by a variety of intracellular sulfhydryl reagents, including methanethiosulfonates, metal divalent cations, and oxidizing agents (49). These reagents inhibit ENaC activity from the cytosolic side by inducing long and slowly reversible channel closures. The contribution of the cytosolic C-terminus of $\alpha\beta\gamma$-ENaC subunits in channel gating still needs to be elucidated.

Modulatory Sites of ENaC Activity

Aldosterone and many other factors, including hormones (*e.g.*, vasopressin, angiotensin II, insulin), extracellular proteases (*e.g.*, kallikrein, channel-activating protease-1), intra- and extracellular ion concentrations (*e.g.*, for Na^+, Ca^{2+}), osmolarity, and the tubular flow rate are known to regulate ENaC activity (1). The regulation of renal sodium transport by ENaC may occur directly either at the level of the single channel by changing the Po present in the apical membrane or at the level of the number (N) of active channels expressed at the cell surface by changing the rate of insertion or retrieval from the cell surface. These two ways of regulation of ENaC activity at the cell surface are not mutually exclusive. In the case of aldosterone-dependent stimulation of Na^+ transport, the insertion of additional ENaC subunits into the apical membrane is supported by immunohistochemical studies (11,12) and single-channel recordings show that ENaC are more active at the cell surface (20,24). Aldosterone regulation of ENaC likely involves both effects on N and Po.

The SGK1 kinase, a member of the PKB/Akt family of serine/threonine kinases, is rapidly induced by aldosterone in ASDN model epithelia *in vitro* (50,51) as well as in the kidney *in vivo* (52,53). Co-expression of ENaC with SGK1 in heterologous expression systems profoundly increases ENaC-mediated Na^+ currents (50,51,54) and ENaC cell surface expression. The regulation of ENaC by SGK can be mediated either directly by phosphorylation of targeted amino acid residues in the ENaC sequence (55) or indirectly by phosphorylating ENaC regulatory proteins. In heterologous expression systems, the effect of SGK1 on ENaC can be mediated by phosphorylation of the ubiquitin-ligase *Nedd4-2* that regulates ENaC activity at the cell surface (56,57).

Nedd4-2 is a ubiquitin ligase that contains in its sequence four WW domains (W for tryptophane) that bind proline-rich ligands. Proline-rich motifs are found in the C-terminus of the $\alpha\beta\gamma$-ENaC subunits, in particular a PP.Y sequence (PY motif) that represents a characteristic pattern for WW peptide ligands. These PPP.Y sequences in the β- and γ-ENaC subunits are the target sequence for mutations that cause Liddle's syndrome (pseudohypoaldosteronism) (58–60). These mutations increase the number of channel molecules at the cell surface as well as the channel activity (Po effect) when expressed in *Xenopus* oocytes (61). Mutations of the PY motif clearly prolong the half-life of the channel at the cell surface as a result of an impaired internalization of ENaC. Two mechanisms have been proposed to explain the cell surface retention of ENaC: First, mutations in the PY motif impair interactions with the binding partner Nedd4-2; because Nedd4-2 catalyzes the attachment of ubiquitin moieties on the channel for endocytosis and degradation, mutations in the PY motif alter this process (62). Second is a defect in the clathrin-mediated endocytosis of ENaC, because the PY motif sequence also contains two adjacent endocytic signals (63). Although these proposed mechanisms are not mutually exclusive, it remains that in heterologous expression systems, ENaC is ubiquitinated, and multiple lysine residues in the N-termini of α (K47 and K50) and γ subunits (K6 to K13) are substrates for attachment of ubiquitin moieties (64). How mutations in the PY motif increase the Po remains to be established. There is presently no good evidence that the C-terminus of ENaC subunits directly participates in channel gating, but it remains possible that the protein interaction with the PY motif of ENaC downregulates the channel (Po effect) before internalization.

Because the PY motif in the C-terminus of ENaC subunits regulates ENaC stability and activity at the cell surface in heterologous expression systems, studies have addressed the question of whether this PY motif represents a final target sequence on ENaC for the aldosterone signaling pathway. In their early report on patients with pseudohypoaldosteronism, Liddle *et al*. (65) clearly showed that these patients retain their ability to respond to aldosterone by reducing their urinary fractional excretion of sodium to almost zero. More recently, single-channel studies in mouse models of Liddle syndrome (pseudohypoaldosteronism) that lack the PY motif in the β-ENaC subunit show that the ENaC response to elevation in plasma aldosterone level is drastically increased in Liddle mice compared with wild-type mice (66). In a cortical CD cell line that expressed ENaC wild-type or mutants with mutations in the PY motif, the rate of increase in Na^+ transport induced by aldosterone during the early response was similar (67). These observations suggest that the PY motif regulates ENaC activity at the cell surface independent of a stimulation of the aldosterone signaling pathway.

Regarding the possibility that SGK1 directly phosphorylates ENaC, the cytosolic C-terminus of the mammalian α-ENaC subunit contains consensus sites for phosphorylation by SGK. The mutation of the serine 621 (rENaC sequence) prevents the effect of SGK1 that recruits more active channel at the cell surface, suggesting that S621 of α-ENaC also participates in the control of ENaC expression at the cell surface (55).

Extracellular trypsin and proteases CAP1 (prostasin), CAP2,

CAP3, and TMPRSS3 activate ENaC (68,69). In addition, the serine protease inhibitor aprotinin reduces the transepithelial sodium transport in an amphibian kidney cell line. In airway epithelial cell lines, elastase, another serine protease, activates ENaC, and a decrease in the expression of prostasin reduces the amiloride-sensitive Na^+ current (70,71). Clearly, the Po of ENaC is increased by extracellular proteases, an effect on the channel that is independent of changes in intracellular Ca^{2+} or activation of G protein–coupled receptors (72,73). In addition, maturation of ENaC involves proteolytic cleavage of the α- and γ-ENaC subunits by furin, a proprotein convertase that also cleaves prohormones, coagulation factors, or receptors at the cell surface (74). The furin-dependent cleavage of ENaC seems to correlate with a higher channel activity at the cell surface, and the relation between ENaC cleavage and the activation of the channel was confirmed in furin-deficient cells (74).

ENaC contains several consensus motifs for furin or trypsin cleavage in the extracellular loop. Furin cleavage of α- and γ-ENaC involves multiple cleavage motifs in the extracellular loop after the first transmembrane segment, suggesting the presence in this region of important functional domains that control ENaC activity (74). These cleavage sites do not overlap with sites for trypsin or serine protease action, which remain to be precisely identified.

Other modulatory sites are likely located in the extracellular loop. For instance, mutations of histidine residues at corresponding positions in the extracellular loop of α- and γ-ENaC differentially modulate ENaC self-inhibition by extracellular Na^+ ions (75). Thus, the extracellular loop may function as an extracellular sensor for Na^+ ions to adapt channel activity with the extracellular concentration of Na^+ ions; in addition, the extracellular loop of ENaC may function as a target for an extracellular signaling cascade involving proteases, necessary for the activation of the channel.

Conclusion

The identification of structural domains that are important for basic channel function (*e.g.*, translocation of Na^+ ions across the membrane) or for the modulation of ENaC activity at the cell surface (N or Po effects) is essential for our understanding of the cellular and molecular mechanisms involved in ENaC regulation by hormones and other intracellular/extracellular factors. In addition, this knowledge about the structure and the function of ENaC will certainly be helpful in interpreting physiologic and pathophysiologic consequences of ENaC genetic variants on Na^+ handling by the ASDN.

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